Identification of Human Liver Cytochrome P450 Enzymes Involved in the Metabolism of SCH 530348 (Vorapaxar), a Potent Oral Thrombin (PAR1) Receptor Antagonist

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Abbreviations used are: P450, cytochrome P450; HPLC, high performance liquid chromatography; FSA, flow scintillation analyzer; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TCR, total chromatographic radioactivity; TRA, thrombin receptor antagonist
ABSTRACT

Vorapaxar (SCH 530348), a potent oral thrombin receptor (PAR-1) antagonist, is being developed as an antiplatelet agent for patients with established vascular disease. The objective of this study was to identify the human liver cytochrome P450 enzyme(s) responsible for the metabolism of SCH 530348. Human liver microsomes metabolized SCH 530348 to M19, an amine metabolite formed via carbamate cleavage, and M20 (monohydroxy-SCH 530348). Recombinant human CYP3A4 exhibited the most activity (11.5% profiled radioactivity) for the formation of M19 followed by markedly less substrate conversion with CYP1A1 and CYP2C19. Trace levels of M19, a major excreted human metabolite, were detected with CYP1A2, CYP3A5 and CYP4F3A. Formation of M19 by human liver microsomes was inhibited 89% by ketoconazole (IC$_{50}$ 0.73 μM), 34% by tranylcypromine and 89% by anti-CYP3A4 monoclonal antibody. There was a significant correlation between the rate of M19 formation and midazolam 1'-hydroxylation ($r = 0.75$) or M19 formation and testosterone 6β-hydroxylation ($r = 0.92$). The results of screening, inhibition and correlation studies confirmed that CYP3A4 is the major P450 enzyme responsible for M19 formation from SCH 530348. In contrast, formation of M20, a major circulating human metabolite at steady state, was primarily catalyzed by CYP3A4 and CYP2J2. M20 is pharmacologically equipotent as SCH 530348 while M19 is an inactive metabolite. Formation of M20 by human liver microsomes was inhibited 89% by ketoconazole, 75% by astemizole (CYP2J2 inhibitor) and 43% by CYP3A4 monoclonal antibody. These results suggest that CYP3A4 and CYP2J2 are both involved in the formation of M20 metabolite.
INTRODUCTION

Vorapaxar; SCH 530348 [ethyl[(1R,3aR,4aR,6R,8aR,9S,9aS)-9-[(E)-2-[5-(3-fluorophenyl)-2-pyridinyl]ethenyl]-dodecahydro-1-methyl-3-oxonaphtho[2,3-c]furan-6-yl]carbamate] (Figure 1), an analog of the natural product himbacine, is a potent antagonist of protease-activated receptor 1 (PAR-1), the primary thrombin receptor on human platelets. Vorapaxar is under investigation for the treatment and prevention of cardiac events in patients with acute coronary syndrome (ACS) and those with prior myocardial infarction (MI) or stroke, as well as in patients with existing peripheral arterial disease. Thrombosis caused by a ruptured or eroded atherosclerotic plaque may result in partial or complete occlusion to coronary and other small arteries. This process is the final underlying mechanism of ACS, including acute myocardial infarction (AMI), and is a major contributing process in ischemic stroke (Yeghiazarians et al., 2000; Davies et al., 1976; Lip et al., 2002).

Oral antiplatelet drugs are at the forefront of antithrombotic agents under investigation for chronic use because antiplatelet drugs are of proven benefit to subjects with unstable angina, MI, stroke, peripheral artery disease, and conditions with high risk of embolism (Dogné et al., 2002; Baigent et al., 2002). Thrombin is the most potent activator of human platelets, stimulating them primarily through the interaction with PAR-1 (Anderluh and Dolenc, 2002; Andersen et al., 1999). Thus, a PAR-1 antagonist is anticipated to demonstrate a higher level of efficacy than either aspirin or adenosine diphosphate (ADP) antagonists. The effects of aspirin, ADP-receptor antagonists, and PAR-1 antagonists are also expected to be complementary because they inhibit different molecular targets on the platelet. With the failure of the oral GpIIb/IIIa antagonists in clinical trials to date, the issue of inter-individual variability in response and resistance to the effects of certain antiplatelet agents (e.g. aspirin), and evidence to suggest that generation of thrombin continues during long-term treatment with antiplatelet agents (Eikelboom et al., 2002), there exists a need for
additional therapies for chronic use. Vorapaxar is being developed to address this need.

The safety and tolerability of vorapaxar was recently demonstrated in patients undergoing non-urgent Percutaneous coronary intervention (PCI) or coronary angiography with planned PCI (Becker et al. 2009).

SCH 530348, administered as a bisulfate salt, is rapidly absorbed and highly bioavailable (>90%) following oral administration (unpublished data). Although SCH 530348 is the major circulating drug-derived component after a single dose in plasma (essentially 100%), it is slowly eliminated, mainly as metabolites, in urine and feces (unpublished data). Parent drug is not eliminated in urine and less than 2% of orally administered SCH 530348 is eliminated unchanged in the feces. The drug is primarily eliminated as the amine metabolite (M19) formed via carbamate cleavage. Minor amounts of mono- and dihydroxy-metabolites and glucuronide and sulfate conjugates are also formed. However, after multiple dose administrations, a hydroxylated metabolite, M20 becomes a predominant circulating metabolite and represents ~23% of the parent drug.

The appreciable accumulation of M20 (M+16 metabolite), an equipotent active plasma metabolite in human was only uncovered after long-term clinical dosing with vorapaxar. The objective of this in vitro study was to identify the cytochrome P450 enzyme(s) capable of metabolizing SCH 530348. Since the in vitro conversion of SCH 530348 to M20 was initially too low, the enzymology of M20 was determined using unlabeled SCH 530348 and then quantitatively studied using a highly sensitive LC-MS/MS method.
MATERIALS AND METHODS

Chemicals. Glucose-6-phosphate (G-6-P) dehydrogenase, monosodium D-glucose-6-phosphate, NADP, magnesium chloride, Trizma base, ammonium acetate and quinidine were purchased from Sigma-Aldrich (St. Louis, MO). Ketoconazole was purchased from Oxford Biomedical Research inc., (Oxford, MI). HPLC grade acetonitrile acetic acid, and methanol were purchased from Fisher Scientific (Fair Lawn, NJ); distilled water was prepared using a Milli-Q water purification system from Millipore (Bedford, MA); and Tris-buffer was purchased from BD Biosciences (Woburn, MA). Unlabeled SCH 530348 and M20 were obtained from Schering-Plough, Kenilworth, NJ. Radiolabeled SCH 530348 (\(^{14}\text{C}\)), radiochemical purity > 97%, specific activity 126 µCi/mg, Figure 1) was prepared by the Radiochemistry Group at Schering-Plough Research Institute (SPRI, Kenilworth, NJ). Single donor (H0029), pooled human liver microsomes (n = 10), intestine and lung microsomes and a Reaction Phenotyping Kit were purchased from XenoTech, LLC (Lenexa, KS). P450 SUPERSOMES™ and CYP3A4 monoclonal antibodies were purchased from BD Biosciences (Woburn, MA).

Incubations with Human Liver Microsomes and S9. Pooled human liver microsomes (1 nmol P450/ml) were incubated with \(^{14}\text{C}\)-SCH 530348 (1-50 µM) for 120 min in the presence of an NADPH-generating system as described previously (Ghosal et al, 2006, 2007). All incubation mixtures contained 3 mM magnesium chloride in 0.5 ml of 100 mM potassium phosphate buffer, pH 7.4. Prior to the addition of drug, the incubation mixture was preincubated for 2 min at 37°C. Reactions were initiated by the addition of drug or NADP\(^+\), allowed to proceed for 120 min at 37°C, and then terminated by the addition of 0.5 ml of methanol. The incubation mixtures were vortexed and centrifuged (10,000g) at 4°C for 10 min and the supernatants analyzed by HPLC-FSA. Only samples from substrate concentrations of 10 and 50 µM were analyzed by LC-MS. Boiled human liver microsomes and incubations without
NADPH served as negative controls. $^{14}$C-SCH 530348 (10µM) was incubated with human liver S9 (1.6 mg protein/ml) using the procedures described above.

**Optimization and Kinetic Parameters using Human Liver Microsomes from a Donor with High CYP3A4 Activity.** Initial incubations of $^{14}$C-SCH 530348 with pooled human liver microsomes showed a low level of conversion to metabolites. Therefore, all subsequent incubations were performed using human liver microsomes from a single donor (H0029) with high CYP3A4 activity. In vitro incubations of $^{14}$C-SCH 530348 with human liver microsomes were performed using various cytochrome P450 concentrations (0.25 to 1 nmol P450/ml) with 10 µM $^{14}$C-SCH 530348 for 30, 60, 90, and 120 min. Substrate concentrations of 1 µM to 100 µM were used subsequently to optimize the concentration of the drug and to calculate Km and Vmax. Reactions were initiated and terminated as described before. The quantitation of M19 (confirmed by LC-MS) was based on radiometric detection and a standard curve (linear least square fit) of seven substrate concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µM).

**Screening of Human P450 SUPERSOMES™.** Screening of human P450 SUPERSOMES™ (CYP1A1, CYP1A2, CYP2A6, CYP1B1, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4F2, CYP4A11, CYP4F3A, CYP4F3B, and CYP4F12) was initially conducted using a constant amount of cytochrome P450 (0.2 nmol/ml) and $^{14}$C-SCH 530348 (10 µM) for 120 min. The screening was also performed under in vitro steady-state conditions of 25 µM $^{14}$C-SCH 530348 (~Km concentration). All incubations were performed in 100 mM potassium phosphate buffer, pH 7.4 as described in the previous section (HLM section). For CYP2C9 and CYP2A6 SUPERSOMES™, incubations were performed in 100 mM Tris-buffer (pH 7.4) as recommended by the supplier. Insect microsomes without cDNA of human P450 were used as control. For LC-MS analysis, supernatants were concentrated under nitrogen at room temperature. Since the rate of M20 formation was low in human liver microsomes and below the detection limit in some cases, kinetic parameters were not determined for M20 formation.
Kinetic parameters of CYP3A4 SUPERSOMES™ were determined as described previously. Km and Vmax were determined using substrate concentrations from 1 to 100 μM.

**Inhibition with Selective P450 Inhibitors and Inhibitory Antibodies.**
Inhibition of SCH 530348 metabolism was initially evaluated using selective chemical inhibitors of cytochrome P450 enzymes (ketoconazole for CYP3A4, quinidine for CYP2D6, sulfaphenazole for CYP2C9, omeprazole / ticlopidine / tranylcypromine for CYP2C19, orphenadrine / ticlopidine for CYP2B6, astemizole for CYP2J2, α-naphthoflavone for CYP1A1/2, and inhibitory monoclonal antibodies against CYP2B6 and CYP3A4. Human liver microsomes (pooled or individual donor, 1 nmol/ml) were pre-incubated separately with various inhibitors for 15 min at room temperature followed by the addition of buffer, cofactor, and substrate (10 and 25 μM 14C-SCH 530348) for M19 formation. The inhibition of M20 formation was also studied with 25 μM unlabeled SCH 530348. All incubations were performed as described previously. Incubation volumes were 0.5 ml and the final concentration of the organic solvents in the incubation system was less than 1% (v/v).

Inhibition of 14C-SCH 530348 metabolism to M19 was further evaluated using selective chemical inhibitors of cytochrome P450 enzymes (ketoconazole for CYP3A4, ticlopidine for CYP2B6/CYP2C19 and tranylcypromine for CYP2C19). Human liver microsomes from donor H0029 (0.5 nmol/ml) were pre-incubated separately with each of the inhibitors for 15 min at room temperature followed by the addition of buffer, cofactor and substrate (25 μM 14C-SCH 530348). All incubations were performed as described above. The IC50 of ketoconazole (0.1-2 μM) for M19 formation was determined using human liver microsomes with high CYP3A4 activity under the conditions described previously.

**Correlation Analysis.** A Reaction Phenotyping Kit consisting of 10 individual human liver microsomal preparations from individual donors was used for correlation analysis. The ability of human liver microsomes from each donor
to metabolize SCH 530348 to its metabolite M19 was correlated with the P450-specific enzyme activities for each sample. The assays were performed as described previously with 25 μM substrate and an incubation period of 120 min. Since the rate of M20 formation was low in human liver microsomes and below the detection limit in some cases, correlation study was not performed for M20 formation.

Detection and Identification of In Vitro Metabolites of SCH 530348.

Samples from the initial microsomal incubations were analyzed for the identification of metabolites using a LC-MS system comprised of a TSQ Quantum mass spectrometer (Thermo Electron Corp., San Jose, CA) operated in a positive electrospray ionization mode, an Alliance 2695 HPLC module (Waters Corp., Milford, MA), and a model C525F00 flow scintillation analyzer (FSA, PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). A Luna Phenyl-Hexyl 250 x 4.6 mm, 5 μm particle size (Phenomenex, Inc., Torrence, CA) column connected to a Luna Phenyl-Propyl 4.0 x 3.0 mm, 5 μm particle size guard column was used for metabolite profiling analyses. The mobile phase, which consisted of (A) 95% 20 mM ammonium acetate (pH adjusted to 6.0 by glacial acetic acid) and 5% acetonitrile, and (B) 95% acetonitrile and 5% 20 mM ammonium acetate (pH adjusted to 6.0 by glacial acetic acid), was maintained at a constant flow rate of 1 ml/min. The temperature of the column was kept at 40°C. Separation was achieved using programmed linear changes in mobile phase composition starting with 10% of B for 2 min, increasing to 50% from 2 to 25 min, increasing to 98% from 25 to 52 min, and then maintaining B at 98% from 52 to 57 min. The HPLC column effluent was split so that 18-21% of the flow was analyzed by MS and 79-82% diverted to the FSA. The mass spectrometer was operated in positive electrospray ionization mode, with the spray voltage set at 3.7-4.8 KV, the tube lens offset at 29-133 V, the capillary temperature at 300°C, the sample flow rate at 0.21 ml/min and the sheath and auxiliary gases at 35-50 and 15-25 arbitrary units, respectively.

M19 was the primary in vitro metabolite observed following incubation of 14C-SCH 530348 with human liver microsomes and P450 SUPERSONES™. The
quantitative determination of M19 was performed using an HPLC system equipped with both UV and FSA comprised of an Alliance Model 2690 HPLC (Waters Corp.), a 996 Photodiode Array Detector (Waters Corp), a 500TR flow scintillation analyzer (PerkinElmer). The analytical column, mobile phase and parameters for gradient elution of parent drug and metabolite(s) were as described in the previous section.

**Sample analysis for Metabolite M20.** Since the enzymology involved in the formation of M20 from SCH 530348 was examined using non-radiolabeled SCH 530348, its disappearance and the formation of M20 were measured using an LC-MS/MS method. \(^{[13\text{C}_6]}\) SCH 530348 and \(^{[13\text{C}_6]}\) M20 were used as internal standards. A Waters Acquity UPLC (Milford, MA, USA) system and an Agilent Zorbax SB-C8 2.1x50 mm column packed with 1.8 µm particles (Wilmington, DE, USA) maintained at 45°C were used for chromatographic separation of drug-derived material. The HPLC system was operated at a constant total flow of 0.5 ml/min in gradient mode. Mobile phase A and B consisted of 0.1% formic acid in water (v/v) and methanol (v/v), respectively. The initial mobile phase consisted of 35% B and was maintained at this ratio for 1.0 min. The mobile phase was changed from 35% to 60% B in 0.1 min, to 75% B from 1.1 to 2.5 minutes, and changed from 75% to 95% B from 2.5 to 2.6 minutes. The mobile phase composition was maintained at 90% B until 3.25 minutes, and then at 3.3 minutes the mobile phase changed from 95% B to the initial condition of 35% B to re-equilibrate the system for subsequent sample injections. The total HPLC run time is 4.0 minutes. Partial loop injection (5 µL) was used to load samples onto the column. The syringe was washed immediately after sample injection with 500 µL of strong wash solvent (0.1/95/5 formic acid/acetonitrile/water) and 500 µL weak wash solvent (25:75 methanol/water).

A Waters Quattro Ultima triple quadrupole mass spectrometer (Milford, MA, USA) equipped with an electrospray ionization (ESI) probe was operated in the positive ion mode for sample analysis. The capillary voltage was set at 4.0 kV and the cone voltage at 35 V. The ion source temperature was set 125°C.
and the desolvation temperature at 350°C. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The ion transitions of 493.2>447.2 (SCH 530348), 499.2>453.2 ([13C6] SCH 530348), 509.2>447.2 (M20 metabolite of SCH 530348), and 515.2>453.2 ([13C6] M20) with collision energy of 30 eV and dwell time of 50 ms were used in the study. A diverting valve was used to direct the flow to waste for the first 1.5 minutes to reduce the potential for ion source contamination.

RESULTS

Incubation with Pooled Human Liver Microsomes and S9. The metabolic conversion of 14C-SCH 530348 to metabolites was low (3-6%) following incubation of the drug (1, 10, 50 µM) with human liver microsomes and S9 (data with 1 and 50 µM are not shown). LC-MS analysis demonstrated that SCH 530348 was converted primarily to an amine metabolite (M19) by carbamate cleavage (Table 1). The structure of M19 was confirmed by LC-MS/MS and retention time matching with reference standard. In the absence of the NADPH-generating system, or with boiled microsomes, or boiled S9, from no to very trace levels of M19 were formed (data not shown).

Optimization and Kinetic Parameters using Human Liver Microsomes from a Donor expressing High CYP3A4 Activity. Human liver microsomes from a single donor expressing high CYP3A4 activity (H0029) were incubated with 10 µM 14C-SCH 530348 at various concentrations of cytochrome P450 (0.25 to 1 nmol/ml) for various time periods (30 to 120 min). The P450 concentration of 0.5 nmol/ml and an incubation time of 120 min were found to be optimal for the assay (data not shown). The effect of various substrate concentrations on the metabolite formation (M19) including their kinetic parameters are shown in Table 2. The Km for formation of M19 was 23.5 µM while its Vmax was 57.9 pmol/nmol P450/min (Table 2). A substrate concentration of 25 µM was chosen for further experiments with human liver microsomes considering the linearity, percent of conversion and detection sensitivity for M19. Representative radiometric profiles of metabolites from incubation of SCH 530348 (25 µM) with human liver microsomes...
are presented in Figure 2. Metabolite M20 formation was below the detection limit of the radiometric detector. Therefore, a more sensitive LC-MS/MS system was used for quantitation of M20. In the absence of the NADPH-generating system, no metabolite formation was observed (data not shown). The total recovery of injected radioactivity from the HPLC column was 92%.

**Incubation of SCH 530348 with Microsomes from Human Liver, Intestine and Lung tissues.** Incubation of unlabeled SCH 530348 with microsomes from human liver, intestine and lung tissues showed that M20 was generated in all microsomal fractions and its formation is highest in the liver>intestine>pulmonary tissues (trace level) (Figure 3). Following incubation of SCH 530348 (25 µM) with human liver microsomes, the rate of M19 formation (~30 pmol/nmol P450/min, not shown) was much higher compared to the rate of M20 formation (~0.4 pmol/nmol P450/min) (Figure 4A and Figure 4B). Experimental variability for the incubations was 2-6%.

**Screening with cDN-A-Expressed Human P450 SUPERSOMES™.** In vitro screening of 14C-SCH 530348 (10 µM) with recombinant human P450 SUPERSOMES™ showed that only CYP1A1, CYP1A2, CYP2C19, CYP3A4, and CYP3A5 yielded metabolites (mainly M19), suggesting possible involvement of these enzymes in the metabolism of SCH 530348. CYP3A4 exhibited the most activity (109.8 pmol/nmol P450/min) followed by markedly less substrate conversion with CYP2C19, CYP3A5, and CYP1A1 (Figure 4A insert). The screening was repeated under steady state condition (25 µM) and the results confirmed that CYP1A1, CYP2C19, CYP3A4, and CYP3A5 yielded M19 (Figure 4A). As expected, CYP3A4 exhibited the most activity (11.5% profiled radioactivity) followed by markedly less substrate conversion by CYP1A1, CYP2C19 and CYP3A5 (Figure 4A). Trace levels of M19 were also detected following incubation of 14C-SCH 530348 with CYP1A2, and CYP4F3A. The formation of radioactive metabolites with recombinant CYP1A1, CYP1A2, CYP2C19, CYP3A4, CYP3A5, and CYP4F3A suggested involvement of these enzymes in the metabolism of SCH 530348.
The metabolite profiles obtained in various incubations are provided in Table 1. For most of the analyzed samples, the conversion (1-6%) was represented by various amounts of an amine (M19, m/z = 421) and monoxy (M20a, m/z = 509) metabolites. CYP3A4 showed significantly higher conversion (27.5%), ~22.1% of which is represented by M19. Therefore, M19 represents 80.4% of all metabolites catalyzed by CYP3A4. Low amounts (≤1%) of three other oxidative metabolites M20a, M22 (m/z = 509), and a monoxy-M19 (M18, m/z = 437) were also detected. Trace levels of two additional monoxy-SCH 530348 metabolites (M23 and M24, m/z = 509) were detected by LC-MS/MS in selected incubations.

Optimization with human CYP3A4 SUPERSOMES™ was performed with 25 μM 14C-SCH 530348 at various concentrations of cytochrome P450 (0.1 to 0.3 nmol/ml) and for various time periods (30 to 120 min). A P450 concentration of 0.2 nmol/ml and incubation time of 120 min was found to be optimal for the assay (data not shown). Km for the formation of M19 from CYP3A4 SUPERSOMES™ was 22.9 μM while its Vmax was 166 pmol/nmol P450/min (Table 2). Similar Km values for M19 formation determined from incubation with human liver microsomes (23.5 μM) and CYP3A4 (22.9 μM) strongly suggest the involvement of CYP3A4 in its formation from SCH 530348. A representative radiometric profile of metabolites from incubation of 14C-SCH 530348 (25 μM) with CYP3A4 SUPERSOMES™ is presented in Figure 2.

The accumulation of M20, an active metabolite in human plasma was only established after chronic administration of vorapaxar. The amount of circulating M20 formed was too low for the detection by a flow scintillation analyzer following a single radioactive dose of vorapaxar to humans (unpublished data). Therefore, the in vitro enzymology of M20 was investigated separately with unlabeled compound using a highly sensitive LC-MS/MS method. Screening of unlabeled SCH 530348 with human CYP SUPERSOMES™ showed that CYP2J2 is the major enzyme generating M20 followed by CYP3A4 (Figure 4B). Other CYPs generated M20 in trace levels. However, the rate of M19 formation (109.8
pmol/nmol P450/min) was much higher compared to the rate of M20 formation (0.7 pmol/nmol P450/min) mediated by CYP3A4. The rate of M20 formation by CYP2J2 was 30.6 pmol/nmol P450/min.

Further screening of M20 (25 µM) with human liver microsomes and 19 CYPs following 120 min Incubation showed that M20 was further metabolized by CYP3A4 and CYP2J2 to downstream metabolites M16 (m/z 523) and M19 (m/z 421) (not shown). Proposed P450-mediated in vitro biotransformation pathways for SCH 530348 is provided in Figure 8.

**Inhibition of M19 with Selective P450 Inhibitors.** The results of chemical inhibition studies using pooled human liver microsomes with selective chemical inhibitors showed that ketoconazole (a CYP3A4 inhibitor) at 2 µM, inhibited the formation of M19/M20a by 74% (Figure 5A). Tranylcypromine (50 µM) and orphenadrine (300 µM) inhibited M19/M20a formation by 16 and 41%, respectively. In contrast, sulfaphenazole (3 µM), quinidine (5 µM), omeprazole (10 µM), and α-naphthoflavone (10 µM) showed no inhibition. Since orphenadrine, a reported CYP2B6 inhibitor, showed 41% inhibition, CYP2B6-specific antibody was used to explore the contribution of CYP2B6 in the metabolism of SCH 530348. CYP3A4-specific inhibitory monoclonal antibody inhibited M19 by 89% whereas the CYP2B6-specific inhibitory antibody had no effect (Figure 5A). Experimental variability was 2-7%. These study results indicate that the effect of orphenadrine may be due to inhibition of CYP3A4. Guo et al (1997) has reported that orphenadrine inhibits multiple P450 enzymes including CYP3A4 in human liver microsomes. Similarly, our studies suggest that CYP3A4 is inhibited by orphenadrine while CYP2B6 has minimal effects on vorapaxar metabolism.

The results of the inhibition studies using human liver microsomes expressing high CYP3A4 activity showed that ketoconazole (2 µM) inhibited the formation of M19 by 89% (Figure 5A). The IC50 value of ketoconazole was 0.73 µM (Figure 6). These inhibition studies suggest that CYP3A4 is primarily responsible for the metabolism of SCH 530348. Tranylcypromine at a higher concentration of 100 µM and ticlopidine (20 µM) inhibited M19 formation by 34%,
and 5%, respectively (Figure 5A). Ticlopidine is an inhibitor of CYP2B6 and CYP2C19 (Turpeinen et al.). The very low level of inhibition by ticlopidine and no metabolite formation by CYP2B6 SUPERSOMES™ again suggests no involvement of CYP2B6 in the metabolism of SCH 530348. However, moderate inhibition by tranylcypromine suggests the minor involvement of CYP2C19.

Inhibition of M20 with Selective P450 Inhibitors – Investigation of the effect of ketoconazole and astemizole on the formation of M20 from CYP3A4, CYP2J2 SUPERSOMES™ and human liver microsomes showed that ketoconazole inhibited M20 formation in human liver microsomes and CYP3A4 by ~90% and in CYP2J2 by ~51% (Figure 5B). The present study with rCYP3A4 and rCYP2J2 demonstrated that ketoconazole is a potent inhibitor of CYP3A4, and a moderate inhibitor of CYP2J2 (Figure 7). Astemizole inhibited M20 formation in human liver microsomes and CYP3A4 by ~76% and in CYP2J2 by ~87%. Hence astemizole is an inhibitor of both CYP2J2 and CYP3A4 (Figure 7). These data suggested that both CYP3A4 and CYP2J2 were involved in the formation of M20. In human intestine, ketoconazole and astemizole inhibits M20 by 80 and 77%, respectively, suggesting its potential formation via intestinal CYPs (CYP3A4 and CYP2J2).

In human liver microsomes, CYP3A4-monoclonal antibody inhibited M20 by ~43% suggesting the involvement of CYP3A4 (Figure 5B). This data also suggests that ~43% of M20 formation is catalyzed by CYP3A4.

Correlation Analysis. The formation rate of M19 from 14C-SCH 530348 was measured in each of the 10 human liver microsomal samples provided in the Reaction Phenotyping Kit. These values were then correlated with the biochemical activity data provided with the kit. Since the biochemical activities are mediated by specific P450 enzymes, a high correlation would suggest that similar enzymes were involved in the formation of metabolite(s) from SCH 530348.

The highest correlation (r) between the Reaction Phenotyping Kit assay data (n = 10) and the formation of M19 was noted for midazolam 1′-hydroxylation (r = 0.75) and testosterone 6β-hydroxylation (r = 0.92, Table 3) which are
catalyzed by CYP3A4 and CYP3A4/5, respectively. There was poor correlation between the formation of M19 and phenacetin O-deethylation mediated by CYP1A2 ($r = 0.22$) and S-mephenytoin 4′-hydroxylation mediated by CYP2C19 ($r = 0.22$) suggesting that CYP1A2 and CYP2C19 have minor involvement. Results of correlation analysis between the enzyme activities and M19 formation confirmed that SCH 530348 is metabolized to M19 primarily by CYP3A4 in human liver microsomes.

**DISCUSSION**

Clinical studies have shown that vorapaxar is extensively metabolized in humans. The major route of elimination of the drug is via an amine metabolite (M19) formed by carbamate cleavage. However, after multiple dose administrations, metabolite M20 becomes a major (>10% total) circulating metabolite when steady state for the parent is achieved. Minor amounts of mono- and dihydroxy-metabolites are also formed (unpublished data). In order to better predict the potential for possible drug-drug interactions following co-administration of SCH 530348 with other drugs, this study was undertaken to characterize the CYP enzymes responsible for the metabolism of vorapaxar. Characterization of the CYP enzyme(s) responsible for the metabolism of SCH 530348 was accomplished by “reaction phenotyping”, including correlation analysis with a panel of characterized microsomal preparations, chemical inhibition, and the use of cDNA-expressed human CYP enzymes.

Initially, $^{14}$C-SCH 530348 was incubated with pooled human liver microsomes and S9 in the presence of an NADPH-generating system to identify in vitro metabolites of SCH 530348. These in vitro incubations demonstrated that SCH 530348 was metabolized primarily to M19 and trace amounts of several other oxidative metabolites. The conversion to M19 was relatively low (<6%) and the level of M20 was below the detection limit. Preliminary identification of the CYP450 enzymes involved in the metabolism of SCH 530348 using screening of 17 recombinant human P450 SUPERSOMES™ suggested the possible involvement of CYP1A1, CYP1A2, CYP3A4, CYP3A5, and CYP 2C19 in the
metabolism of SCH 530348, with M19 being the major metabolite formed under the in vitro conditions employed. CYP3A4 exhibited the highest activity followed by markedly less substrate conversion with CYP2C19, CYP3A5, and CYP1A1.

Reaction conditions were subsequently optimized using human liver microsomes from a single donor (H0029) expressing high CYP3A4 activity. However, the level of M20 was still near or below the detection limit (flow scintillation analyzer). Optimal conditions were determined to be a P450 concentration of 0.5 nmol and an incubation time of 120 min. Further screening of 19 recombinant human P450 SUPERSOMES™ showed that CYP1A1, CYP1A2, CYP2C19, CYP3A4, CYP3A5, and CYP4F3A yielded M19. As with the initial screening, CYP3A4 exhibited the most activity (11.5% profiled radioactivity) followed by markedly less substrate conversion by CYP1A1 and CYP2C19. The Km for the formation of M19 from human liver microsomes was 23.5 µM and from CYP3A4 SUPERSOMES™ was 22.9 µM. The comparable Km values for M19 formation using human liver microsomes (23.5 µM) and CYP3A4 (22.9 µM) strongly suggest the involvement of CYP3A4 in its formation (Table 2).

Chemical inhibition studies also suggested the involvement of CYP3A4 in the formation of M19. Inhibition studies done with pooled human liver microsomes demonstrated that ketoconazole (2 µM), a selective CYP3A4 inhibitor, inhibited the formation of M19/M20a by 74%. The results of additional inhibition studies using human liver microsomes with high CYP3A4 activity showed that ketoconazole (2 µM) inhibited the formation of M19 by 89%. The IC50 value of ketoconazole was determined to be 0.73 µM. These inhibition studies suggest that CYP3A4 is primarily responsible for the metabolism of SCH 530348.

Orphenadrine, a reported CYP2B6 and CYP3A4 inhibitor (Guo et al, 1997), inhibited M19/M20a formation 41%. However, further studies using monoclonal antibodies demonstrated that while CYP3A4-specific inhibitory monoclonal antibodies inhibited M19 by 89%, CYP2B6-specific inhibitory antibodies showed no inhibition. These results suggested that CYP3A4 is primarily responsible for the metabolism of SCH 530348 to M19 and that CYP2B6 has no contribution.
Tranylcypromine (100 μM), a CYP2C19 inhibitor, and ticlopidine (20 μM), an inhibitor of CYP2B6 and CYP2C19 (Turpeinen et al.), inhibited M19 formation by 34%, and 5%, respectively. The very low level of inhibition by ticlopidine again suggested the lack of involvement of CYP2B6 in the metabolism of SCH 530348. However, moderate inhibition by tranylcypromine suggested minor involvement of CYP2C19.

The results of correlation analysis between the enzyme activities and M19 formation confirmed that SCH 530348 is metabolized to M19 primarily by CYP3A4 in human liver microsomes. The highest correlation with the formation of M19 was noted for midazolam 1'-hydroxylation \((r = 0.75)\) and testosterone 6β-hydroxylation \((r = 0.92)\) catalyzed by CYP3A4 and CYP3A4/5, respectively. There was no correlation between the formation of M19 and phenacetin O-deethylation mediated by CYP1A2 \((r = 0.22)\) and S-mephenytoin 4'-hydroxylation mediated by CYP2C19 \((r = 0.22)\). The poor correlation suggests that CYP1A2 and CYP2C19 may have only minor involvement in the formation of M19. The proposed biotransformation pathway of SCH 530348 to M19 (Figure 8) involves oxidation primarily via CYP3A4 of the secondary carbon of the ethyl group followed by loss of C₂H₄O and then CO₂. Compared to CYP3A4, the contributions of CYP1A1, CYP1A2, CYP2C19, and CYP3A5 to the biotransformation of SCH 530348 in human liver microsomes are minor.

Screening of SCH 530348 with recombinant human CYP SUPERSOMES™ showed that CYP2J2 is the major enzyme generated M20 followed by CYP3A4. Other CYPs generated M20 in trace levels. In the chemical inhibition study using human liver microsomes (expressing high CYP3A4 activity), formation of M20 was inhibited ~89% by ketoconazole and ~75% by astemizole. Based on the literature and our study, ketoconazole and astemizole both are capable of inhibiting CYP3A4 and CYP2J2 (Matsumoto et al, 2003). Other CYP inhibitors showed little or negligible inhibition, suggesting their role is minor. In human liver microsomes, CYP3A4-monoclonal antibody inhibited M20 by ~43% suggesting the involvement of CYP3A4. This data also suggests that ~43% of M20 formation is catalyzed by CYP3A4 and rest by CYP2J2 and other CYPs. CYP2J2-antibody
is not commercially available to distinguish contribution of CYP3A4 and CYP2J2. These data suggest that both CYP3A4 and CYP2J2 are involved in the formation of M20.

Further screening of M20 (25 μM) with recombinant human CYPs showed that M20 was further metabolized by CYP3A4 and CYP2J2 to downstream metabolites M16 and M19.

The overall role of CYP2J2 in drug metabolism has not been determined to date. Several antihistamine drugs, including terfenadine, ebastine, and astemizole, have been identified as good substrates for CYP2J2, a P450 isoform predominantly expressed in the intestine and heart tissues, with low levels in the liver (Delozier et al., 2007). In fact, strong overlap in substrate recognition by CYP2J2 and CYP3A4 was observed among all the newly identified CYP2J2 substrates.

In human, the abundance of CYP3A4 is 82% in intestine and 40% in liver while the abundance of CYP2J2 is 1.4% in intestine and 1-2% in liver (Paine et al, 2006). Hence we can conclude that CYP3A4 will have major contribution in the formation of M20. Contribution of CYP2J2 would likely be less than that for CYP3A4. In a clinical drug interaction study with ketoconazole (CYP3A4 and CYP2J2 inhibitor) the combined effect was to increase the exposure only two-fold.
ACKNOWLEDGEMENTS

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Authorship Contributions

Participated in research design: Ghosal, Lu, Gao, Ramanathan, Chowdhury, Kishnani and Alton.
Conducted experiments: Ghosal, Lu, Ramanathan, Penner, and Gao.
Contributed new reagents or analytic tools: Ghosal and Lu.
Performed data analysis: Ghosal, Lu, Penner, Gao, Ramanathan, and Chowdhury.
Wrote or contributed to the writing of the manuscript: Ghosal, Gao, Chowdhury, Kishnani and Alton.
REFERENCES


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LEGENDS FOR FIGURES

Figure 1. Chemical structure of SCH 530348, (*) denotes site of [14C]-label

Figure 2. HPLC Profiles of radioactivity following In vitro incubation of SCH 530348 (25 µM) with human liver microsomes from a single donor H0029 (top panel) or CYP3A4 SUPERSOMES™ (bottom panel) with an NADPH-generating system.

Figure 3. Incubation of SCH 530348 (25 µM) with human liver microsomes (HLM), human intestine microsomes (HIM), and human lung microsomes (HPM) in the presence and absence of NADPH. Values represent mean of n=2.

Figure 4. 4A. Screening of 14C-SCH 530348 (25 µM) for M19 Formation with P450 SUPERSOMES™ together with an NADPH-generating system. Insert: Incubation with 10 µM 14C-SCH 530348.

4B. Screening of unlabeled SCH 530348 (25 µM) for M20 Formation (Monohydroxy-SCH 530348) with P450 SUPERSOMES™ together with an NADPH-generating system.

Figure 5. (A) Effect of chemical inhibitors and inhibitory monoclonal antibodies on the metabolism of SCH 530348 (10 and 25 µM) to M19 using pooled human liver microsomes (■) or human liver microsomes expressing high CYP3A4 (H0029). (B) Effect of chemical inhibitors and inhibitory monoclonal antibodies on the metabolism of unlabeled SCH 530348 (25 µM) to M20 using microsomes from human liver with high CYP3A4 activity (HLM) or intestine (HIM).

Figure 6. Determination of IC50 value of ketoconazole for the formation of M19 from 14C-SCH 530348 in human liver microsomes with high CYP3A4 activity. Standard error ±0.05.

Figure 7. Contribution of CYP3A4 and CYP2J2 using cDNA Expressed Recombinant Enzymes and Human Liver Microsomes (HLM): Inhibition of M20 Formation by Ketoconazole (5 µM) and Astemizole (100 µM). Values represent mean of n=2.

Figure 8. Proposed P450-mediated in vitro biotransformation pathways for SCH 530348.
<table>
<thead>
<tr>
<th>Metabolite label</th>
<th>Name</th>
<th>m/z</th>
<th>Percent of total radiochromatographic radioactivity (%TCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>HLM</td>
</tr>
<tr>
<td>M18</td>
<td>Monooxy SCH530348 M18</td>
<td>437</td>
<td>-</td>
</tr>
<tr>
<td>M19</td>
<td>M19</td>
<td>421</td>
<td>5.12</td>
</tr>
<tr>
<td>M20a</td>
<td>Monooxy SCH530348 M20</td>
<td>509</td>
<td>-</td>
</tr>
<tr>
<td>M22</td>
<td>Monooxy SCH530348 M22</td>
<td>509</td>
<td>-</td>
</tr>
<tr>
<td>Parent</td>
<td>SCH530348 Parent</td>
<td>493</td>
<td>92.7</td>
</tr>
<tr>
<td>M23</td>
<td>Monooxy SCH530348 M23</td>
<td>509</td>
<td>-</td>
</tr>
<tr>
<td>M24</td>
<td>Monooxy SCH530348 M24</td>
<td>509</td>
<td>-</td>
</tr>
</tbody>
</table>

HLM: Human liver microsomes  
HLS9: Human liver S9  
(-) = Not detected  
a: BIT = Below integration threshold of flow scintillation analyzer detector but detected by MS  
b: M19 and M20a were not separated in the radiochromatogram. However, based on LC-MS response of each, the contribution to the radioactive peak was determined.
Table 2: Kinetic parameters for the formation of M19 following incubation of \(^{14}\)C-SCH 530348 with human liver microsomes and human CYP3A4 SUPERSOMES™

<table>
<thead>
<tr>
<th>Kinetic Parameters (^{a})</th>
<th>Human Liver Microsomes</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km (µM)</td>
<td>23.5 ± 1.91(^{b})</td>
<td>22.9 ± 1.84</td>
</tr>
<tr>
<td>Vmax (pmol/nmol P450/min)</td>
<td>57.9 ± 1.75</td>
<td>166 ± 4.92</td>
</tr>
<tr>
<td>Vmax/Km (µl/nmol P450/min)</td>
<td>2.46</td>
<td>7.25</td>
</tr>
</tbody>
</table>

Human liver microsomes with high CYP3A4 activity (H0029)

a: Kinetic parameters were determined by GraFit 5.0.1 program.

b: ± Standard error

c: Intrinsic clearance
Table 3 Correlation (r) values between M19 formation rates from SCH 530348 and P450 enzyme specific activities

<table>
<thead>
<tr>
<th>P450 Enzyme Specific Reaction</th>
<th>P450 Involved&lt;sup&gt;a&lt;/sup&gt;</th>
<th>M19 (r value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin O-Deethylation</td>
<td>CYP1A2</td>
<td>0.22</td>
</tr>
<tr>
<td>Coumarin 7-Hydroxylation</td>
<td>CYP2A6</td>
<td>0.38</td>
</tr>
<tr>
<td>Bupropion Hydroxylation</td>
<td>CYP2B6</td>
<td>0.14</td>
</tr>
<tr>
<td>Paclitaxel 6α-Hydroxylation</td>
<td>CYP2C8</td>
<td>0.13</td>
</tr>
<tr>
<td>Diclofenac 4'-Hydroxylation</td>
<td>CYP2C9</td>
<td>0.11</td>
</tr>
<tr>
<td>4'-Hydroxylation</td>
<td>CYP2C19</td>
<td>0.22</td>
</tr>
<tr>
<td>S-Mephenyton 4'-Hydroxylation</td>
<td>CYP2D6</td>
<td>0.68</td>
</tr>
<tr>
<td>Dextromethorphan O-Demethylation</td>
<td>CYP2E1</td>
<td>0.44</td>
</tr>
<tr>
<td>Chlorzoxazone 6-Hydroxylation</td>
<td>CYP3A4/5</td>
<td>0.75&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Midazolam 1'-Hydroxylation</td>
<td>CYP3A4/5</td>
<td>0.92&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Testosterone 6β-Hydroxylation</td>
<td>CYP4A11</td>
<td>0.10</td>
</tr>
</tbody>
</table>

SCH 530348 = 25 μM

<sup>a</sup> Enzyme activities are from Reaction Phenotyping Kit (n = 10)
<sup>b</sup> p = 0.0127
<sup>c</sup> p = 0.0001
Fig 2.
Fig. 3

Formation of M20 (nM)

- HLM+NADPH
- HLM-NADPH
- HIM+NADPH
- HIM-NADPH
- HPM+NADPH
- HPM-NADPH
Fig 5A

- **α-Naphthoflavone (10 µM)**
- **Orphenadrine (300 µM)**
- **Sulfaphenazole (3 µM)**
- **Omeprazole (10 µM)**
- **Tranylcypromine (50 µM)**
- **Tranylcypromine (100 µM)**
- **Quinidine (5 µM)**
- **Ketoconazole (2 µM)**
- **Ticlopidine (20 µM)**
- **Antibody Control**
- **MAb/CYP2A4**
- **MAb/CYP2B6**

% Inhibition

- 10 µM
- 25 µM (H0029)
Fig 5B

- Sulfaphenazole (3 µM)
- Quinidine (5 µM)
- Ketoconazole (5 µM)
- Ticlopidine (20 µM)
- Astemizole (100 µM)
- Antibody Control
- MAb-CYP3A4

% Inhibition
IC_{50} = 0.73 \mu M

Fig 6
Fig 7

% Inhibition

CYP3A4 (Ketoconazole) 90%
CYP2J2 (Ketoconazole) 50%
HLM (Ketoconazole) 90%
CYP3A4 (Astemizole) 70%
CYP2J2 (Astemizole) 80%
HLM (Astemizole) 80%
FIG 8

Mono-oxy metabolites M20a/M22/M23/M24

SCH 530348

M19

CYP 3A4/CYP2J2

CYP 3A4

CYP 3A4/CYP2J2

M20

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